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Mycobacteria: Laboratory Methods for Testing Drug Sensitivity and Resistance

G. CANETTI,[1] S. FROMAN,[2] J. GROSSET,[3] P. HAUDUROY,[4] MILOSLAVA LANGEROVÁ,[5] H. T. MAHLER,[6] GERTRUD MEISSNER,[7] D. A. MITCHISON[8] & L. ŠULA[9]

In its seventh report, published in 1960, the WHO Expert Committee on Tuberculosis "noted the need for international standards for the definition and determination of drug resistance which will permit comparisons to be made from one area to another, and recommended that the World Health Organization take appropriate steps to establish such standards".[10] *Acting on this recommendation, WHO took the first step towards standardization by convening in Geneva, in December 1961, an informal international meeting of specialists in the bacteriology of tuberculosis. At this meeting an attempt was made to formulate prerequisites for reliable sensitivity tests and to specify the technical procedures for them.*

The first part of the present paper is a joint contribution by the participants in the meeting, summarizing the general conclusions reached and recommendations made with regard to tests of sensitivity to the three main antituberculosis drugs—isoniazid, streptomycin and p*-aminosalicylic acid. The other three parts describe, in turn, three different tests for determining drug sensitivity—the absolute-concentration method, the resistance-ratio method and the proportion method—that are generally considered to give reasonably accurate results.*

I. INTRODUCTION[11]

Since the introduction of potent antimicrobial drugs, great advances have been made in the control of tuberculosis and other infectious diseases. However, with few exceptions, the activity of these drugs has been limited by the appearance of strains of bacteria which are capable of growth in the presence of unusually high concentrations of the drugs. Tubercle bacilli have been found that are resistant to each of the drugs introduced for the treatment of tuberculosis. Resistant strains can be produced from cultures in the laboratory and have also been obtained from experimental animals and from

[1] Chef de Laboratoire, Service de la Tuberculose, Institut Pasteur, 25 rue du Docteur Roux, Paris 15e, France.

[2] Director, Microbiological Research Laboratory, Olive View Hospital, Olive View, Calif., USA.

[3] Assistant, Institut Pasteur, 25 rue du Docteur Roux, Paris 15e, France.

[4] Directeur, Institut de Bactériologie, d'Hygiène et de Virologie, Faculté de Médecine, 19 avenue César-Roux, Lausanne, Switzerland.

[5] Research Assistant, Tuberculosis Research Institute, Šrobárova 48, Prague 10, Czechoslovakia.

[6] Chief Medical Officer, Tuberculosis, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland.

[7] Tuberculosis Research Institute, Borstel über Bad Oldesloe, Germany.

[8] Director, Unit for Research on Drug Sensitivity in Tuberculosis (Medical Research Council of Great Britain), Postgraduate Medical School of London, Ducane Road, London W.12, England.

[9] Chief, Department of Bacteriology and Epidemiology, Tuberculosis Research Institute, Šrobárova 48, Prague 10, Czechoslovakia. Formerly Medical Officer, Tuberculosis, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland.

[10] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **195**, 11.

[11] Prepared by the participants in an informal meeting of Advisers on Laboratory Methods for the Drug Sensitivity/Resistance Determination of Mycobacteria, held in Geneva from 4 to 7 December 1961: Dr G. Canetti, Dr S. Froman, Professor P. Hauduroy, Dr Miloslava Langerová, Dr H. T. Mahler, Professor Gertrud Meissner, Dr D. A. Mitchison and Dr L. Šula.

patients treated with the drug concerned. In the majority of instances, these strains arise by the selective growth of small numbers of resistant mutant bacilli present in the bacterial population before it comes into contact with the drug. In a few instances, tubercle bacilli may be naturally resistant to a drug before they have ever come into contact with it. Thus the bovine type may be naturally resistant to *p*-aminosalicylic acid (PAS) and to pyrazinamide.

The importance of bacterial resistance has been reviewed by the WHO Expert Committee on Antibiotics which, in its second report,[1] made the following observation:

" Bacterial resistance to antibiotics is the principal obstacle to their successful therapeutic use. When resistance develops during a course of treatment, it may deprive an antibiotic of its proper therapeutic effect in the patient being treated. More important in the long run is the effect on the general community, since the elimination of sensitive strains and the dissemination of resistant ones leads to a situation in which many infections are resistant *ab initio* and alternative treatment must be adopted. For this reason, the estimation of bacterial sensitivity or resistance to antibiotics has assumed great importance. Such estimations are an essential prerequisite for the rational use of antibiotics and for preserving the efficacy of this important group of therapeutic substances."

The widespread use of two or more drugs given simultaneously in treatment has markedly reduced the emergence of drug resistance during treatment, but, in the event of failure to use such combined therapy in the most efficient manner, patients continue to produce large numbers of drug-resistant organisms in their sputum. Other patients may become infected with these resistant bacilli and are then far less likely to respond satisfactorily to the drug concerned.

A major problem in any control programme of tuberculosis based on the application of mass chemotherapy is the danger of the widespread dissemination of drug-resistant tubercle bacilli. There are reasons to believe that strains resistant to isoniazid and streptomycin did not occur in the community prior to the introduction of these drugs, and it is probably true that there was very little resistance to PAS. Surveys of the prevalence of primary drug resistance in many countries have indicated that from 3% to 15% of strains from previously untreated patients may be resistant to one or more of the three standard drugs. It is possible that the prevalence of drug-resistant strains may be increasing, particularly in those countries where the usual treatment of patients is unsatisfactory.

Laboratory tests of the sensitivity of tubercle bacilli to chemotherapeutic drugs serve three main purposes: first, they can be used as a guidance in the choice of the first course of chemotherapy to be given to the patient. Secondly, they may be of value in confirming that drug resistance has emerged when a patient has failed to show a satisfactory bacteriological response to treatment, and may guide the choice of a further course of treatment with different drugs. Thirdly, they may be employed to estimate the prevalence of primary and of acquired drug resistance in a community. For each of these purposes, it is of great importance to use a reliable technique in performing the test. Unfortunately, many different techniques and methods of interpreting the results are in use (Rist & Crofton [2]). It is probable that many of these techniques fail to distinguish with any precision between sensitive and resistant organisms.

Before prerequisites for reliable sensitivity tests can be formulated and technical procedures for them laid down, agreement must be reached on the definition of the terms " sensitive " and " resistant ". In our opinion, " sensitive " and " resistant " strains of *Mycobacterium tuberculosis* may be defined as follows: " Sensitive " strains are those that have never been exposed to the main antituberculosis drugs (" wild " strains) and that respond to these drugs, generally in a remarkably uniform manner. " Resistant " strains are those that differ from sensitive strains in their capacity to grow in the presence of higher concentrations of a drug. This definition of resistance is based on the laboratory response; strains that are resistant in this sense do not *necessarily* fail to respond to the usual doses of the drug in the lesions of the patient. However, a diminished clinical response is likely to occur whenever resistance is demonstrated in the laboratory, even though the extent or degree of that resistance is small.

In the following discussion of possible methods of determining drug sensitivity, we have borne in mind the need for a test that is accurate, reproducible, economical and rapid. Consideration has

[1] WHO Expert Committee on Antibiotics (1961) *Wld Hlth Org. techn. Rep. Ser.*, **210**, 3.

[2] Rist, N. & Crofton, J. (1960) *Bull. int. Un. Tuberc.*, **30**, 2.

been given only to the three main antituberculosis drugs—isoniazid, streptomycin and PAS—although we fully realize that accurate tests for resistance to the minor drugs are also of considerable importance.

STANDARDIZATION OF A SENSITIVITY TEST

Techniques for performing sensitivity tests vary widely in different laboratories, both in the methods employed and in the interpretation of the results. It seems probable that some of these techniques classify a proportion of wild strains as resistant or, conversely, a proportion of resistant strains as sensitive. A satisfactory method should yield a minimum of misclassification in either of these directions. For this reason, we recommend that any method employed should be standardized in terms of the response of a sample of wild strains. Since there is some evidence that strains of *Myco. tuberculosis* vary in their sensitivity to chemotherapeutic agents according to the country of their origin, it would be desirable to obtain these sample strains from one or more countries where the prevalent organisms have been adequately studied and where the therapeutic response of patients to the drug concerned is known. Standardization using samples of wild strains may present problems owing to difficulties of storage and transportation of the strains.[1] A standard sensitive strain (possibly H37Rv) may be employed as a sub-standard if its response to the drug concerned has been compared in one laboratory with a suitable sample of wild strains by the method to be employed.

TYPES OF SENSITIVITY TEST IN CURRENT USE

Sensitivity tests in current use may be classified, in the first place, as direct or indirect. In *direct tests*, the sputum homogenate or other pathological material is cultured directly on drug-containing medium. The tests are performed only on those specimens that contain adequate numbers of acid-fast bacilli as shown by direct smear examination (at least one bacillus in ten high-power microscopic fields), since results may not be reliable when the growth on culture is scanty. The exact procedures to be adopted are also determined in most methods by the number of bacilli seen in direct smear examinations.

In many laboratories, direct sensitivity tests are not performed—partly, because it is not considered practical to examine smears as a routine before inoculation of the culture medium and, partly, because direct sensitivity tests performed by some techniques are not considered as reliable as those done by the indirect method. Whatever method is used, indirect tests will be necessary for cultures where the direct smear examination was negative or only scantily positive.

The main reason for the use of direct tests is that they reduce the time between obtaining the specimen from the patient and reading the sensitivity result from about seven weeks, the time necessary for the indirect test, to four weeks. With certain patients—for instance, those with progressive disease about to receive a re-treatment course of chemotherapy—it is important to obtain the results of a reliable test as soon as possible; direct sensitivity tests are of particular value in these circumstances.

In *indirect tests*, the drug-containing medium is inoculated from the primary diagnostic culture. Indirect tests may be classified into three main categories: (*a*) the absolute-concentration method; (*b*) the resistance-ratio method; and (*c*) the proportion method. Examples of method (*a*) have been described by the United States Veterans Administration [2] as well as by Meissner in Part II of this paper (see page 570). Method (*b*), which is used in investigations under the auspices of the Medical Research Council of Great Britain, is described by Mitchison in Part III of this paper (see page 573) and method (*c*) is described by Canetti & Grosset in Part IV (see page 574).

Each of these three indirect tests may be adapted for use as a direct test. In the absolute-concentration and resistance-ratio methods, additional controls are necessary; several different inocula of the standard strain should be employed to correspond in bacillary content to the expected range of positivity of the test material. An advantage of the proportion method is that this elaboration is unnecessary since the direct and indirect methods are virtually identical.

[1] Šula, L., Sundaresan, T. K. & Langerová, M. (1960) *Bull. Wld Hlth Org.*, **23**, 635.

[2] United States Veterans Administration, Department of Medicine and Surgery (1960) *Tuberculosis laboratory methods for the V. A. Armed Forces Cooperative Study on the Chemotherapy of Tuberculosis*, Washington, D.C., p. 9.

RELATIVE MERITS OF THE METHODS

A satisfactory test by the absolute-concentration method is obtained only:

(*a*) if the inoculum employed is adequately standardized in size; and

(*b*) if the critical concentration of the drug has been standardized for the laboratory and for the method concerned by reference to an adequate sample of wild strains.

In such a test, it is usually necessary to standardize the method before it is brought into general use, and control studies must be done at intervals to see that no alterations in the inoculum or the medium have occurred. Without adequate standardization it has proved particularly unsatisfactory to transfer the findings obtained in one laboratory to another laboratory.

The satisfactory performance of the resistance-ratio method also depends upon adequate standardization of the size of the inoculum of both the test strain and the standard strain, but it is not necessary to define the critical drug concentration since a running control is provided by the standard strain. This method requires slightly more medium than the absolute-concentration method. Furthermore, the results obtained are very slightly less accurate and it is necessary to re-test strains with doubtful results a little more frequently.

The proportion method has the advantage that standardization of the size of the inoculum is not of critical importance since the inoculum size is known from the viable counts carried out in the test. The results are possibly influenced to a certain extent by the degree of dispersion of the inoculum suspension, so that it is of particular importance to adhere to the exact procedure for preparing the suspension. It is also necessary to determine the correct critical concentration to be used in the test in the same manner as for the absolute-concentration method. Although no comparison of this and the other two methods has been published, the proportion method may perhaps be slightly more accurate than the others since adjustment is made for an important source of variation in the results—the size of inoculum. The test takes slightly longer to carry out than the other methods, and requires the performance of quantitative techniques.

RECOMMENDATIONS

We consider that the best type of sensitivity test is a fully quantitative determination in which the proportion of organisms capable of growth on medium containing a wide range of drug concentrations is known. This type of test would provide full information both on the degree of the resistance and on the proportion of resistant organisms at each drug concentration. However, since such a test requires large amounts of medium and is time-consuming, it cannot be recommended as a routine procedure. It will be appreciated that each of the foregoing methods is a compromise in which one or other of the assessments of the results of a test is given the greatest prominence. A fully quantitative test should be employed whenever the circumstances permit.

We are unable to recommend a single standard method for adoption, but are agreed on the following technical features of sensitivity tests:

1. The medium to be employed should be Löwenstein-Jensen without potato starch, described as the medium of the International Union against Tuberculosis (IUT medium).[1] Löwenstein-Jensen medium is widely used. The omission of potato starch does not appear to influence the results of sensitivity tests and the IUT medium is simpler to prepare.

2. Solutions of the chemotherapeutic drugs can be dissolved in sterile distilled water and added to the medium without further sterilization. However, if stock solutions of the drugs are to be stored, they should be sterile, either as a result of preparing them from sterile ampoules of the drug or by filtration through a membrane, sintered-glass or candle filter, but not by Seitz filtration.

3. In reporting the concentrations of drug in the medium (particularly streptomycin or dihydrostreptomycin), the concentration should be that present in the medium before inspissation. No account should be taken of possible inactivation during inspissation or storage.

4. Drug-containing medium can be stored at 4°C for a period not exceeding three months. Such medium should not be stored at room temperature because of appreciable destruction of certain drugs.

5. Attention is again drawn to the fundamental importance of standardizing all methods of performing sensitivity tests. It would be particularly desirable to achieve standardization (*a*) in recording

[1] Jensen, K. A. (1955) *Bull. int. Un. Tuberc.*, **25**, 89.

the results in terms of the response of wild strains of tubercle bacilli, and (*b*) in respect of the inoculum.

Simple test for isoniazid resistance

We recommend the following simple test for isoniazid resistance:

A representative loopful of the growth on the primary diagnostic culture is taken with a loop of 3-mm external diameter made from thick nichrome wire (for example, 22 SWG [1]). The amount of this growth is about 2 mg (moist weight) visualized as 2 mm^3 on the loop. The loopful of growth is added to a screw-capped bottle or similar container with a volume of approximately 25 ml (for instance, a 1-ounce bottle) containing 12 glass beads (2-3 mm in diameter) and 0.5 ml of sterile distilled water. The bottle is shaken for 10 to 20 seconds by hand and to it is added 9.5 ml of sterile distilled water. As a safety precaution, a fresh cap is put on the bottle, which is then briefly shaken again. From the suspension so prepared a loopful is taken with a loop of 3-mm external diameter made from thin nichrome wire (for example, 27 SWG) and placed on each of three slopes of Löwenstein-Jensen medium without potato starch (IUT medium) containing, respectively, 0, 0.2 and 1.0 μg/ml of isoniazid. The slopes are incubated for four weeks at 37°C and are then read.

The presence of 100 or more colonies on 0.2 or 1.0 μg/ml, or both, of isoniazid is taken as an indication that the strain is resistant to isoniazid.

Circumstances for performance of the simple test for isoniazid resistance or of a more accurate test

We recommend that, wherever possible, sensitivity tests be carried out by one of the three "indirect" procedures considered as accurate. The simple test for isoniazid resistance may be done before the start of antituberculosis chemotherapy as a guidance in the choice of the regimen either for patients who claim not to have received previous chemotherapy or for those whose treatment is being changed following the failure of a previous regimen. If resistance is demonstrated either by one of the accurate methods or by the simple test on a strain obtained from patients who claim not to have received previous chemotherapy, the result should not be taken to indicate that treatment with isoniazid must necessarily be withheld. However, such a result indicates that it is advisable to give additional chemotherapy preferably with at least two drugs other than isoniazid.

In any survey of the prevalence of resistant tubercle bacilli in a country or territory, the sensitivity tests should be carried out, whenever possible, in laboratories equipped and staffed for the purpose of one of the recommended accurate procedures. However, preliminary information, which must be treated with reserve, can be obtained by the performance of the simple test for isoniazid resistance.

Future areas for research

With regard to particularly important subjects for further research, we make the following recommendations:

1. Attempts should be made to improve the standardization of sensitivity tests and to test their applicability in different laboratories. Of importance is the question of the standardization of the media employed, including the investigation of lyophilized or dried medium. It is recommended that a comparison be made between different carefully selected methods in each of several laboratories. For this purpose a single set of strains should be tested in each laboratory. The methods themselves must be strictly adhered to in each laboratory.

2. Further research is necessary on rapid methods of sensitivity testing, since the direct tests mentioned in the present paper require a period of four weeks before the result is available.

3. The influence of primary resistance on the therapeutic progress of patients under chemotherapy is of great interest in assessing the clinical significance of laboratory tests. In such studies both the degree of resistance and the proportion of organisms that are resistant in the bacterial population should be determined.

4. Studies should be conducted in different territories on the prevalence of infections due to drug-resistant organisms in patients who claim not to have received previous chemotherapy. If possible, attempts should be made to subdivide such patients into those who failed to disclose previous chemotherapy at the time when the specimen was obtained and those who have true primary resistance.

5. Further information on the spread of isoniazid-resistant organisms within the community is urgently required.

[1] British Standard Wire Gauge.

II. THE ABSOLUTE-CONCENTRATION METHOD [1]

BASIC OBSERVATIONS

The absolute-concentration technique of determining mycobacterial drug sensitivity has been in use for some years throughout the world. The author presents here a description of the method, as modified by her in respect of some important points.

Preparation and checking of uniform drug concentrations

The method requires the preparation of uniform drug concentrations in the culture medium and their careful checking in each new batch of media. This checking should not be confined only to the concentrations used for testing the strains under investigation. In addition, each drug must be tested for the minimum concentrations in which the reference strain H37Rv or other susceptible strains isolated from untreated patients are able to multiply. This point is particularly important since the international control strain, H37Rv, is a little more resistant to isoniazid, and in particular to PAS, than the so-called " wild " strains.

Choice of appropriate size of inoculum

The method also requires particular care in the choice of the appropriate size of inoculum. Since the composition of a culture of *Myco. tuberculosis* is not uniform with regard to the proportion of resistant and sensitive bacilli but corresponds statistically to a normal distribution (bell-shaped) curve, there will be for each strain some concentrations to which all the micro-organisms will be resistant, and others to which only a smaller or larger proportion of them will be resistant. The result, of course, will depend to a large extent on the size of the inoculum.

Resistance on the part of the micro-organisms is clinically significant when at least 1% of the total bacterial population develops at the so-called critical concentrations, that is, the weakest concentrations at which susceptible bacilli are unable to grow in the presence of the drug. Any smaller proportion of resistant micro-organisms has no clinical significance.

The inoculum must therefore be selected so that it is certain to show 1% of resistant micro-organisms; to obtain, for example, growth of about 50-100 bacilli, the inoculum must contain at least 5000-10 000 bacilli. If the inoculation is performed with a loop (85-100 loops = 1 ml), 1 ml of the bacterial suspension should therefore contain 500 000-1 000 000 bacilli. If a 0.1-ml capillary pipette is used for the inoculation, the number of micro-organisms in the suspension should not be more than 50 000-100 000 per ml.

Since it is not possible to determine the viable counts for each culture to be tested in the course of routine drug-sensitivity determinations, the size of the inoculum must be chosen with the greatest care.

TECHNIQUE OF RESISTANCE DETERMINATION

Culture medium

Löwenstein-Jensen medium with 0.75% glycerol is used; 5 ml of medium are added to a rimless test-tube or screw-capped test-tube (80 mm long, 27 mm in diameter) and the medium is then coagulated in a slanting position in flowing steam at 82-85°C for 40-60 minutes, the time of coagulation being counted from the moment when the above temperature is reached. If an autoclave is used, it is possible first to create a vacuum and then to introduce hot steam; in this case, the time for coagulation is reduced to about 20 minutes.

During coagulation, the rimless test-tubes should be plugged with cellulose; for storing in the refrigerator at +4°C, they must be hermetically closed with rubber bungs to prevent desiccation. If screw-capped tubes are used, the cap should be only half screwed down during coagulation and screwed down completely only when the tubes are taken out.

After inoculation, the rubber bungs in the test-tubes should be replaced with plugs that will allow entry of a little air. For this purpose, rubber bungs with a spiral perforation or with a strand of silk threaded through the middle may be employed.

Preparation of the drug dilutions

For isoniazid, streptomycin (i.e., dihydrostreptomycin base), and PAS (i.e., pure *p*-aminosalicylic acid), 1% stock solutions are prepared in distilled water and the necessary amounts of drug are calculated as follows: 1 g of isoniazid = 1 g; 1 g of

[1] Prepared by Professor Gertrud Meissner.

dihydrostreptomycin = 1.17 g of dihydrostreptomycin sulfate; 1 g of PAS = 1.38 g of sodium PAS. From these stock solutions are prepared the dilutions to be added to the culture medium in a proportion of 1 : 10 before coagulation. The dilution used should therefore be 10 times more concentrated than the desired final dilution in the culture medium.

For thioacetazone [1] and ethionamide [2] (1g = 1 g), a 1% solution in ethylene glycol is prepared. From this stock solution, the dilutions are prepared in distilled water, the pipettes and the distilled water being previously warmed to prevent precipitation of the drug. Immediately after the dilutions have been prepared, they should be added to the egg medium (9 ml of culture medium plus 1 ml of dilution), which is then mixed well and transferred as quickly as possible to the culture tubes. Both drugs remain in solution in the egg medium.

Stock solutions of streptomycin, thioacetazone, and ethionamide can be stored in the refrigerator for a limited time in flasks fitted with rubber stoppers. Stock solutions of PAS should be prepared freshly each time, and the same procedure is recommended also for isoniazid. Final dilutions of all these drugs should be prepared freshly for each batch of medium.

The drugs are added to the culture medium before coagulation.

The drug solutions need not be sterilized by filtration before they are added to the culture medium provided that they have been prepared under sterile conditions and provided that the drug-containing medium is heated during coagulation.

For the determination of drug sensitivity by a simple standard method, the following drug concentrations in the medium are recommended:

Drug	*Drug concentrations (µg/ml)*		*Additional concentrations for reference strains (µg/ml)*	
Isoniazid	0.2	1.0	0.05	0.01
Streptomycin (dihydrostreptomycin sulfate)	5.0	10.0	2.0	1.0
PAS	0.5	2.0	0.2	0.1
Thioacetazone	1.0	5.0	0.5	0.1
Ethionamide	20	50	10	5.0

For each strain tested, two control tubes without any drug should be used.

[1] 4′-formylacetanilide thiosemicarbazone.
[2] 2-(ethyl)thioisonicotinamide.

Preparation of the inoculum

In the case of moist colonies (for example, those grown on Gottsacker egg-yolk medium), the bacilli may be ground with the loop directly against the wall of a test-tube containing about 1 ml of Dubos medium without albumin. If the colonies are drier, they should be ground in a Griffith mortar, also containing Dubos medium without albumin. The adjustment is made by comparing the turbidity of the suspension with that of a standard barium sulfate solution equivalent to 1 mg/ml of wet bacterial mass. The bacterial suspension obtained in this way is diluted 1: 50, that is, two drops (0.1 ml) are added to 4.9 ml of Dubos medium. This gives a suspension containing 0.02 mg of bacteria per ml, i.e., about 200 000-1 000 000 organisms per ml. A loopful (diameter: 3 mm) of the suspension (85-100 loops = 1 ml) containing 2 000-10 000 organisms is used as the inoculum.

The inoculum should never be smaller than this, and may be only slightly larger.

Incubation

The cultures should be incubated for four weeks at 37°C. If the cultures are still not readable, incubation should be prolonged for one to two weeks.

Reading of the cultures

++++ = confluent growth, as in the controls.
+++, ++ = discrete colonies according to amount of growth.
\+ = 50-100 isolated colonies.
(+) = 20-49 isolated colonies.

Less than 20 isolated colonies is considered effective inhibition. The result is considered to be negative if the controls produce profuse growth.

A distinction should be made between total resistance (growth as in the controls) and partial resistance (growth markedly less than in the controls). Finally, note should be taken of any case in which there is only a very small number of resistant organisms.

Evaluation of results

For each drug it is necessary to determine the lowest concentration at which the bacilli may no longer be considered susceptible, but are to be regarded as resistant ("critical" concentration).

This concentration should be determined in each laboratory according to the existing experimental conditions, on a series of so-called "wild" strains cultivated side by side with control strains. Under the above-mentioned test conditions, the "critical" concentrations are considered to be: 0.2 μg/ml for isoniazid; 5 μg/ml for streptomycin; 0.5 μg/ml for PAS; 1 μg/ml for thioacetazone; 20 μg/ml for ethionamide. If at these concentrations growth is observed to the extent of more than 20 colonies, the strain is to be considered resistant.

Communication of results

The laboratory report should include the details of the tests and the results of the test for each drug. Sample reports from the Borstel Tuberculosis Research Institute on a susceptible strain (A) and a resistant strain (B) are shown in Table 1.

TABLE 1

DRUG-SENSITIVITY TESTS ON MYCOBACTERIA IN PURE CULTURE ON LÖWENSTEIN-JENSEN MEDIUM

A. Susceptible Strain

Drug concentration in culture medium	Isoniazid	Strepto-mycin	PAS	Thioaceta-zone	Ethion-amide
50 μg/ml	0	0			0
20 ,,					0
10 ,,	0	0	0	0	0
5 ,,		0			++++
2 ,,			0		
1 ,,	0		0	0	
0.5 ,,			0		
0.2 ,,	0				
0.05 ,,	0				
Controls:	++++	++++			

Conclusions: Susceptible to isoniazid, streptomycin, PAS, thioacetazone, and ethionamide

B. Resistant Strain

Drug concentration in culture medium	Isoniazid	Strepto-mycin	PAS	Thioaceta-zone	Ethion-amide
50 μg/ml	+++	+++			0
20 ,,					++++
10 ,,	++++	++++	0	0	++++
5 ,,		++++			++++
2 ,,			0		
1 ,,	++++		++	++++	
0.5 ,,			+++		
0.2 ,,	++++				
0.05 ,,	++++				
Controls:	++++	++++			

Conclusions: Almost completely resistant to 50 μg/ml isoniazid.
Almost completely resistant to 50 μg/ml streptomycin.
Partially resistant to 1 μg/ml PAS.
Completely resistant to 1 μg/ml thioacetazone.
Completely resistant to 20 μg/ml ethionamide.

III. THE RESISTANCE-RATIO METHOD [1]

MEDIUM

Sensitivity tests are done on Löwenstein-Jensen medium without potato starch.[2] The drugs are added before inspissation in the concentrations noted below. The medium is dispensed in about 3-ml amounts in half-ounce (14-ml) screw-capped bottles and is inspissated once for 50 minutes at 85°C.

INOCULUM

The sensitivity tests are set up with an inoculum prepared from the growth on primary diagnostic Löwenstein-Jensen medium slopes, that is to say, they are indirect tests. Tests are set up within two weeks of the slopes becoming positive (usually within three days), and older growths are subcultured if necessary to ensure that the inoculum is composed of young viable organisms.

With a 22 SWG [3] nichrome loop, a representative sweep from the growth is taken on the loop with a volume, judged by eye, of 2 mm^3 (approximately 2 mg moist weight of bacilli). The growth taken on the loop is then discharged into 0.4 ml of sterile distilled water contained in quarter-ounce (7-ml) screw-capped bottles together with six 3-mm glass beads. Standardization of the size of the inoculum is important and depends on estimating the amount of growth on the loop as 2 mm^3. It is advisable that the worker should initially weigh a number of such loopfuls of growth to ensure that there is actually 2 mg of growth on his loop. A suspension is prepared by shaking the quarter-ounce bottle for one minute on a mechanical shaker, and then, with a 3-mm external diameter 27 SWG nichrome loop, a loopful of the suspension is spread on the surface of each slope of the sensitivity test.

A control drug-free slope is set up for each strain tested. The standard sensitive strain, H37Rv, is tested in each set of tests and again within each set if the batch of medium is changed.

INCUBATION AND READING OF TESTS

The slopes are incubated at 37°C. A reading may be made at two weeks to give a preliminary indication as to the presence of resistant strains, but the final and definitive reading is at four weeks, and a report that a strain is *sensitive* should not be given earlier. For all tests, "growth" is defined as the presence of 20 or more colonies. The resistance ratio (RR) is the minimal concentration inhibiting growth (as defined above) of the test strain divided by the minimal concentration inhibiting growth (as defined above) of the standard sensitive strain, H37Rv, in the same set of tests.

SHORT TEST

After the test has been in use for a period, the range of drug concentrations may be shortened with little loss of information. The concentrations that could be omitted in our tests are indicated with an asterisk below. However, it is possible that slightly different concentrations might be needed in other laboratories. The range required for the test strain is determined by the variation in the minimal inhibitory concentration (MIC) of H37Rv, and by the need to determine a resistance ratio of 2 or less for sensitive strains and a resistance ratio of 8 or more for resistant strains. Thus, if H37Rv is inhibited by either 8 or 4 μg/ml streptomycin in a series of batches of tests, then it would be necessary to have 8 μg/ml as the lowest streptomycin concentration in the test strain range (test strain MIC = 8 μg/ml; H37Rv MIC = 4 μg/ml; RR = 2 or less) and 32 μg/ml as the highest concentration (test strain grows on 32 μg/ml; H37Rv MIC = 8 μg/ml; RR = 8 or more).

STORAGE

All stock solutions are normally prepared at monthly intervals and stored at 4°C. Drug-containing media are also stored at 4°C, and can be used for at least two months after preparation.

PROCEDURE WITH DIFFERENT DRUGS

Isoniazid

Stock solutions are prepared in distilled water and sterilized with a membrane, candle or sintered-glass filter, or by very brief autoclaving (5 minutes at 10 pounds per square inch (0.7 kg/cm^2)). Seitz filtration should not be practised as it removes some of the isoniazid.

[1] Prepared by Dr D. A. Mitchison.

[2] Jensen, K. A. (1955) *Bull. int. Un. Tuberc.*, **25**, 89.

[3] British Standard Wire Gauge.

Concentrations in test medium

Test strain: 0.2, 1, 5* and 50* μg/ml isoniazid
H37Rv: 0.025,* 0.05, 0.1, 0.2 and 1* μg/ml isoniazid

* Not necessary in short test. However, the inclusion of 50 μg/ml is helpful in the identification of atypical mycobacteria, since growth of tubercle bacilli on this concentration is usually catalase-negative, whereas that of atypical mycobacteria is often catalase-positive.

Definition of resistance

Sensitive: no growth (less than 20 colonies) on 0.2 μg/ml isoniazid

Resistant: growth on 1 μg/ml isoniazid

Doubtful: growth on 0.2 but not on 1 μg/ml isoniazid

(A doubtful test is repeated from the control slope. If the same reading is obtained, or a more resistant one, the strain is finally classified as resistant. If there is no growth in the repeat test on 0.2 μg/ml, the strain is finally classified as sensitive.)

Streptomycin

Stock aqueous solutions are prepared with aseptic precautions from sterile ampoules of streptomycin sulfate.

Concentrations (before inspissation) in test medium

Test strain: 8, 16, 32, 64* and 1024* μg/ml streptomycin
H37Rv: 2, 4, 8 and 16* μg/ml streptomycin

* Not necessary in short test.

Definition of resistance

Sensitive: a resistance ratio of 2 or less
Resistant: a resistance ratio of 8 or more
Doubtful: a resistance ratio of 4

(A doubtful test is repeated from the control slope. If a resistance ratio of 4 or more is obtained, the strain is finally classified as resistant. If the ratio in the repeat test is 2 or less, the strain is finally classified as sensitive.)

PAS

Stock solutions are prepared from sterile ampoules of sodium PAS dihydrate.

Concentrations in test medium

Test strain: 1,* 2, 4, 8 and 16* μg/ml sodium PAS dihydrate
H37Rv: 0.25,* 0.5, 1 and 2 μg/ml sodium PAS dihydrate

* Not necessary in short test.

Definition of resistance. As for streptomycin.

IV. THE PROPORTION METHOD [1]

PRINCIPLE OF THE METHOD

Every wild strain of tubercle bacilli contains some mutants resistant to antibacterial drugs. The difference between a resistant strain and a susceptible strain is that the *proportion* of resistant bacteria among the total number of bacteria making up the strain is much higher in a resistant strain than in a susceptible one.

If a resistance test is to enable this proportion to be calculated, it must provide information on the *total* number of viable bacteria and on the number of *resistant* bacteria present in the inoculum. This information is obtained by seeding dilutions of the inoculum in tubes containing medium without the drug and in tubes containing medium with the drug. If the dilutions are well chosen, colonies can be obtained in both sets of tubes in numbers that can be counted, and it is easy to deduce from them the proportion of resistant bacteria in the total viable bacterial population.

[1] Prepared by Dr G. Canetti and Dr J. Grosset.

THE STANDARD TEST

Culture medium; concentrations of drugs employed; incorporation of drugs into the medium

Löwenstein-Jensen medium is used for all the resistance tests. The tubes used are 17 mm in diameter and contain 7 ml of medium. The drugs, dissolved in distilled water, are incorporated in the medium before coagulation. In the standard test, media containing the following drug concentrations are used:

Isoniazid: 0.2 μg/ml and 1 μg/ml
Streptomycin: 4 μg/ml and 10 μg/ml
PAS: 0.5 μg/ml and 1 μg/ml

The control medium without the drug is prepared at the same time as the drug-containing media.

In the case of media containing streptomycin, the drug to be incorporated is dihydrostreptomycin sulfate, not streptomycin sulfate. The susceptibility or resistance of strains to these drugs is the same, but the results of the tests are much more consistent when dihydrostreptomycin sulfate is used, because of the

much greater variations in the degree of inactivation of streptomycin sulfate in egg media.

In the case of media containing PAS, the concentrations given here are for *p*-aminosalicylic acid. If a sodium salt of PAS is used to prepare the solution, 1.38 g of the salt are employed for 1 g of *p*-aminosalicylic acid.

After the drug has been added to the medium and after the medium has been distributed in the tubes, the medium is coagulated at 85°C for 50 minutes. The tubes are left at room temperature for 24 hours with cotton-wool plugs and are then covered with rubber caps and put in the refrigerator.

Technique of the indirect test

A spatula measuring 12 mm by 6 mm is used to pick off portions containing the greatest possible number of colonies from the primary culture. The inoculum thus obtained is placed in a spherical flask, 5 cm in diameter, containing 0.5 ml of distilled water and 30 glass beads 5 mm in diameter.

The flask is shaken by hand for 20-30 seconds; 5 ml of water are added. The bacterial suspension is then removed and placed in a tube of 17-mm diameter. The opacity of the suspension is adjusted by the addition of sterile distilled water to correspond to that of a standard suspension containing 1 mg/ml of tubercle bacilli.

Tenfold serial dilutions (10^{-1} mg/ml, 10^{-2} mg/ml, etc., down to 10^{-5} mg/ml) are made from this stock suspension. They are made in distilled water, the liquid being repeatedly aspirated in the pipette in order to ensure a homogeneous mixture and the pipette being changed for each dilution. The dilutions to be inoculated on the media with and without the drug are 10^{-3} mg/ml and 10^{-5} mg/ml. The inoculum for each tube is 0.2 ml. Two tubes of medium without the drug and one tube of medium with the drug are seeded with each dilution. Sixteen tubes in all are used in a standard test for the three major antituberculosis drugs.

After inoculation, the tubes are plugged with cotton-wool but the caps are not replaced. The tubes are put in a stand at a very slight angle from the horizontal and placed in the incubator at 37°C. It is important that the liquid should cover the whole surface of the medium without, however, touching the cotton-wool plug. When the liquid part of the inoculum has evaporated (after 24-48 hours), the tubes are covered with rubber caps and left in the incubator at 37°C until the results are read.

The time needed by an experienced technician to carry out an indirect standard test for the three major antituberculosis drugs is 10-15 minutes.

Technique of the direct test

Any specimen in which microscopic examination demonstrates a sufficient number of bacilli can be used for a direct resistance test. The technique described below is the one applied to sputum.

Two millilitres of sputum are placed in a sterile mortar. An equal quantity of 4% NaOH and 3 drops of tincture of litmus are added. The mixture is triturated with the pestle for one or two minutes and then wrapped in sterile paper and incubated at 37°C for 50 minutes.

A smear of the sputum specimen is prepared beforehand. During incubation of the sputum, the smear is stained by the Ziehl-Neelsen method and examined under the microscope.

1. If the smear is found to contain less than one bacillus per 10 immersion-objective fields, or no bacilli at all, the specimen is subjected to the indirect test described above.

2. If the smear proves to contain at least one bacillus per 10 fields, the specimen is subjected to a direct test, as described below.

The sputum is taken from the incubator after 50 minutes, and *not* centrifuged. It is then neutralized with a few drops of 15% sulfuric acid. The product thus obtained constitutes Dilution 1. Further dilutions are prepared in tenfold serial steps. The dilutions to be used for inoculation depend on the number of bacilli seen under microscopic examination.

1-10 bacilli per 10 immersion-objective fields. Dilution 1 and Dilution 10^{-2} are inoculated on the drug-containing slopes and on the control slopes (without drug).

1-10 bacilli per immersion-objective field. The control tubes and drug-containing tubes are inoculated with Dilutions 1 and 10^{-2}. The control media only are inoculated with Dilution 10^{-3}.

10 bacilli per immersion-objective field. The drug-containing tubes and control tubes are inoculated with Dilutions 10^{-1} and 10^{-3}.

The inoculum for each tube is 0.2 ml. The number of tubes inoculated per dilution and the procedure after inoculation are the same as in the indirect test.

Reading the results ; criteria of resistance

The results are read for the first time on the 28th day. If the results of the test for sensitivity to a given drug are such that, according to the criteria indicated below, the answer is " strain resistant ", no further reading of the test is required. If the strain is shown to be sensitive, a second reading, made on the 40th day, will provide the definitive answer.

In the tubes inoculated with the lowest dilution that is still positive the number of colonies is carefully counted. It is important that even very small colonies should be counted. The number of colonies obtained in the control tubes (average of the two tubes) indicates the number of *viable bacillary units* contained in the inoculum for the corresponding dilution. The number of colonies obtained in the tube containing the drug indicates the number of *resistant* bacillary units contained in the same inoculum.

The ratio between the second figure and the first shows whether the strain is susceptible or resistant. Results are read in the same way in the direct and indirect tests.

The criteria of resistance are as follows:

Isoniazid. Any strain containing at least 1% of bacilli resistant to 0.2 μg/ml of isoniazid or a higher concentration is classified as isoniazid-resistant.

Streptomycin. Any strain containing at least 1% of bacilli resistant to 4 μg/ml of dihydrostreptomycin or a higher concentration is classified as streptomycin-resistant.

PAS. Any strain containing at least 1% of bacteria resistant to 0.5 μg/ml of PAS or a higher concentration is classified as PAS-resistant.

It may happen in a direct test that less than 100 colonies are obtained with the lowest dilution (generally Dilution 1) in the tubes not containing the drug. In such cases, if the tubes containing the weakest concentration of the drug do not yield any colony, an indirect test should be made. The frequency with which this happens varies according to the material studied: it is in the region of 10-15% of tests. It is very rare in the indirect test.

OTHER TYPES OF TEST BY THE PROPORTION METHOD

The standard test described above answers the needs of routine practice. Two other types of test for which a direct or indirect technique can be used can be performed by the proportion method. One is more complex—the "population study test"—while the other is simpler and is known as the " economical test ".

Population study test

For research purposes it may be useful to know what proportion of mutants with very different levels of resistance exists in a strain of *Myco. tuberculosis.* In that case a wider range of concentrations of the drug in the medium must be used. Media containing the following drug concentrations are then recommended:

	μg of drug per ml of medium			
Isoniazid	0.1	0.2	1	5
Dihydrostreptomycin	2	4	10	100
PAS	0.25	0.5	1	10

Furthermore, if an indirect test is being performed, a bacterial dilution of 10^{-1} mg/ml should be seeded as well as the dilutions of 10^{-3} mg/ml and 10^{-5} mg/ml. Forty-two tubes are needed for a test of this kind.

Economical test

It is possible to perform a valid resistance test using a smaller number of tubes than that indicated for the standard test. With this in mind, only one control tube is inoculated with each of the two bacterial dilutions of 10^{-3} mg/ml and 10^{-5} mg/ml. Furthermore, the tubes containing the higher concentrations of the drugs are eliminated in each dilution (the tubes kept are 0.2 μg/ml of isoniazid, 4 μg/ml of dihydrostreptomycin, and 0.5 μg/ml of PAS). The number of tubes needed for the economical test is eight. It should be emphasized that it is impossible in any case to dispense with the inoculation of one or other of the two bacillary dilutions of 10^{-3} and 10^{-5} mg/ml and that the same care should be given to the preparation of these dilutions as in the standard test.

TEST OF RESISTANCE TO " SECONDARY " ANTIBACTERIAL AGENTS

The susceptibility of strains of *Myco. tuberculosis* to what are known as the " secondary " antibacterials (ethionamide, cycloserine, viomycin, kanamycin and thioacetazone is measured in the same way as susceptibility to the major antituberculosis drugs. Information on the concentrations of the drug to be incorporated in the medium and the minimum proportions of resistant bacilli that must be found on the critical concentration of each drug if the strain tested is to be considered resistant can be obtained on request from the authors.

TABLE 2

NUMBER OF MUTANTS RESISTANT TO VARIOUS CONCENTRATIONS OF THE THREE MAJOR ANTITUBERCULOSIS DRUGS FOUND AMONG 10^6 BACILLI OF WILD STRAINS OF *MYCO. TUBERCULOSIS* [a]

A. Isoniazid

Number of resistant mutants	0.1 μg/ml	0.2 μg/ml	1 μg/ml	5 μg/ml
Minimum	2	0.5	0	0
Maximum	180	41	12	7
Average	41	5	3.3	1.5

B. Dihydrostreptomycin

Number of resistant mutants	2 μg/ml	4 μg/ml	10 μg/ml	200 μg/ml [b]
Minimum	100	0.7	0	0
Maximum	100 000	400	12	1
Average	>5 000 [c]	41	2	0.1

C. PAS

Number of resistant mutants	0.25 μg/ml	0.5 μg/ml	1 μg/ml	10 μg/ml
Minimum	1	0.1	0	0
Maximum	11 000	100	32	8
Average	800 [c]	25	4.6	0.7

[a] Drugs incorporated in Löwenstein-Jensen medium before inspissation; results read on the 40th day.

[b] The concentration now advised is 100 μg/ml.

[c] Median value.

CONTROL TESTS TO BE CARRIED OUT IN A LABORATORY PERFORMING SUSCEPTIBILITY TESTS BY THE PROPORTION METHOD

These control tests are necessary in two circumstances: first, when a laboratory begins to use the proportion method and, secondly, every time a new batch of drug-containing media is made in the laboratory or is received from outside.

Preliminary test required when the proportion method is first used

In these circumstances, it is strongly recommended that the method be tried out on a series of five wild strains. These strains should preferably be requested from a reference laboratory; otherwise strains could be used that have been isolated in the laboratory itself from patients who are known for certain never to have been treated with antituberculosis drugs and never to have been in contact with treated tuberculosis patients. These five strains, cultivated on Löwenstein-Jensen medium, are subjected to a special indirect test known as the "control test". The inoculum is obtained and dilutions (down to 10^{-6} mg/ml) are prepared in strict conformity with the method already described above. The concentrations of drugs in the media to be used for the control test are the same as those indicated for the standard test, namely :

	μg of drug per ml of medium	
Isoniazid	0.2	1
Dihydrostreptomycin	4	10
PAS	0.5	1

The following bacillary dilutions are seeded in one tube not containing a drug and in one tube for each drug concentration: 1 mg/ml; 10^{-1} mg/ml; 10^{-2} mg/ml.

The following bacillary dilutions are seeded only in two tubes not containing a drug: 10^{-3} mg/ml; 10^{-5} mg/ml; 10^{-6}mg/ml.

The total number of tubes needed for this control test is 27 per strain. The final results are those read on the 40th day and are expressed as the number of resistant bacilli present among 10^6 viable bacilli of the strain studied.

The figures found for each strain should be compared with those given in Table 2. In at least four of the five strains tested, they should fall, for each drug concentration, between, or very close to, the minimum and maximum values given in the table for the corresponding drug concentration.

Control test required when a new batch of media is prepared or received

In these circumstances, a control test identical with that described above should be performed on *one* wild strain. The same strain should always be used. It should be one of the five strains studied in the preliminary test, preferably one which did not give extreme values for any of the three drugs. Between tests, the strain should be maintained by monthly subculture on Löwenstein-Jensen medium. It is quite unnecessary to perform a control test on a wild strain for each new series of resistance tests.

Note. If the laboratory intends, in certain cases, to use the " population study test ", the complete range of media used in that test should be used for the initial as well as for the routine control tests. The bacillary dilutions to be used for inoculation are those indicated above.

RÉSUMÉ

Depuis l'introduction des médicaments antituberculeux, de grands progrès ont été faits dans la lutte contre la tuberculose et d'autres maladies infectieuses. Cependant, l'activité de ces substances est limitée par l'apparition de souches bactériennes capables de se développer en présence de fortes concentrations de ces médicaments. On connaît maintenant des souches de bacilles tuberculeux résistantes à chacun des principaux médicaments antituberculeux. Il arrive même que certaines souches soient naturellement résistantes à une substance chimique — avant d'avoir été mises en contact prolongé avec elle. C'est ainsi que le bacille tuberculeux bovin peut être naturellement résistant à l'acide *p*-aminosalicylique. On évalue à 3-15% le nombre de souches provenant de malades non traités qui se révèlent résistantes à un ou plusieurs des principaux médicaments antituberculeux (isoniazide, streptomycine, PAS).

Le Comité OMS d'experts des Antibiotiques a estimé que, pour sauvegarder l'efficacité — à l'échelle de l'individu et de la collectivité — des médicaments antituberculeux, il était essentiel de déterminer la sensibilité des souches de bacilles tuberculeux à ces substances. Car, un des problèmes majeurs des campagnes contre la tuberculose fondées sur la chimiothérapie de masse est la dissémination de bacilles résistants aux médicaments.

Les méthodes de détermination et d'appréciation de la sensibilité étant diverses et variées, et leur uniformisation représentant une première étape, fondamentale, vers la connaissance des phénomènes de résistance, un groupe de spécialistes en bactériologie de la tuberculose a été réuni par l'OMS. Cet article donne un aperçu de leurs discussions et un résumé de leurs recommandations.

Les tests de laboratoire relatifs à la sensibilité du bacille tuberculeux aux médicaments ont trois buts principaux: 1) donner une indication pour le premier traitement chimiothérapique à appliquer au malade; 2) confirmer — ou infirmer — l'apparition de la résistance bacillaire lorsqu'un malade n'a pas réagi favorablement au traitement; 3) évaluer l'existence d'une résistance naturelle ou acquise des bacilles tuberculeux disséminés dans la collectivité. Les microbiologistes devraient disposer de techniques sûres. Malheureusement, plusieurs méthodes sont en usage qui manquent de précision et ne permettent pas de distinguer les micro-organismes sensibles des résistants.

En vue de la standardisation des tests de sensibilité, trois types de méthodes ont été proposés:

1. méthode des concentrations absolues
2. méthode des rapports de résistance
3. méthode des proportions.

Ces méthodes sont décrites en détail dans l'article.

Dans leurs recommandations, les participants constatent qu'il n'est pas possible de recommander, à l'issue de cette première réunion, une seule méthode standard, et que chacune des trois méthodes envisagées représente un compromis. Mais quelques caractères généraux doivent être retenus pour les tests de sensibilité.

Le milieu doit être celui de Löwenstein-Jensen, sans fécule de pomme de terre (milieu de l'Union internationale contre la Tuberculose). Les solutions des substances chimiothérapiques doivent être diluées dans l'eau distillée. La concentration (surtout s'il s'agit de streptomycine) sera celle réellement ajoutée au milieu. On ne tiendra pas compte de son éventuelle inactivation partielle pendant la coagulation du milieu. Le milieu peut être conservé à +4°C pendant trois mois au maximum. On cherchera à uniformiser les diverses étapes des tests, en particulier en donnant les résultats par rapport à des souches « sauvages » de bacille tuberculeux et en indiquant la teneur de l'inoculum en bacilles viables.